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Photobiotreatment model (PhBT): a kinetic model for microalgae biomass growth and nutrient removal in wastewater

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This article proposes a kinetic model for wastewater photobiotreatment with microalgae (the PhBT model). The PhBT model for nutrient uptake, coupled with the Verhulst growth model, is a simple and useful tool to describe batch experiments of nutrient removal by microalgae. The model has been validated with experiments of *Chlorella vulgaris* (*C. vulgaris*) grown in wastewater and different synthetic media. The model provided information about nitrogen and phosphorus limitation and their luxury uptake during the test. Productivity observed in synthetic medium (0.17 g SS L^{-1} d^{-1}) was similar to that obtained in nutrient enriched wastewater (0.15 g SS L^{-1} d^{-1}). Biomass productivity of this alga in wastewater and the efficient nutrient removal suggested that *C. vulgaris* could be cultured in wastewater for biomass production while nutrients are reduced from this stream.

Keywords: urban wastewater; nutrient removal; *Chlorella vulgaris*; kinetic model; biomass production

1. Introduction

Eutrophication is defined as 'enrichment of water by nutrients especially compounds of nitrogen and phosphorus, causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms and the quality of the water concerned' [1] and it is one of the primary problems confronting surface waters nowadays [2]. Excessive nitrogen and phosphorus enrichment results in negative impacts on lakes, reservoirs and flowing waters, having biological, economical and physicochemical effects. It is, therefore, necessary to reduce the flow of these substances from anthropogenic sources to natural water bodies. European Directive 98/15/EC [1] concerning urban wastewater treatment set minimum values for total phosphorus and total nitrogen discharges, being 1 mg L^{-1} and 10 mg L^{-1}, respectively.

Microalgae can remove nitrogen and phosphorus from wastewater efficiently and hence have the potential to play an important remediation role during the tertiary treatment of wastewater [3], which has been assessed extensively [4–6]. Concretely, the *Chlorella* genus is very tolerant and can grow successfully in wastewater as a culturing media [7–12].

A literature review of wastewater treatment using microalgae [3] revealed that a huge amount of work has been done; however data treatment of different studies does not follow a common pattern. This leads to a high difficult when interpreting or comparing different works [13–15].

Kinetic models not only provide a basis for reactor design but also for process improvement through facile exploration of diverse environmental and operational conditions. Monod [16] and Droop [17] are the more important equations for kinetic studies with algae. Monod is not reliable in batch conditions because luxury uptake of nutrients, it is the ability to store excess nitrogen and phosphorus. Conversely, using the Droop model is warranted under non-steady-state conditions, however the cell quota (storage capacity of an organism for a specific nutrient necessary for maintenance and growth) of the limiting nutrient in the culture is required and it is not easy to measure. Both models are a function of the limiting nutrient and are not reliable when growth is inhibited by multiple nutrients.

Due to luxury uptake, biomass growth and nutrient uptake are not coupled in batch experiments, so it is difficult having one model for both kinetics. So for practical reasons it may be better using two different models, one for growth and another one for nutrient uptake, sharing some of both models' kinetic parameters. The Verhulst model [18] has been utilized as a fundamental growth model in ecological studies because of its mathematical simplicity and simple biological definition. The Verhulst model only depends on the population level, though accounting for the finiteness of the resource and is much simpler than the Droop and Monod models. In addition, a general kinetic model for degradation processes [19], usually applied for biodegradation of surfactants, can be used to describe the nutrient uptake.

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The aim of this work is adapting a kinetic model for microorganisms substrate uptake [19] to the dissolved nutrient removal of microalgae batch cultures. Furthermore, it proposes the photobiotreatment model (PhBT), which couples the [19] model to the simple and widespread logistic Verhulst model [18] for biomass growth. The parameters of the proposed model will be obtained by fitting the experimental data of microalgae growth and nutrient removal in different culture media to a set of equations. Additionally, growth and nutrient removal kinetics of *Chlorella vulgaris* (*C. vulgaris*) in wastewater and synthetic media are compared.

1.1. Nomenclature

$\delta X(t)/\delta t$: Velocity of microorganisms concentration change ($M\,L^{-3}\,T^{-1}$)
X: Momentary concentration of microorganisms ($M\,L^{-3}$)
X_m: Maximum cell concentration that the system can reach in batch ($M\,L^{-3}$)
X_o: Initial cell concentration ($M\,L^{-3}$)
X_{90}: Biomass value of 90% of X_m ($M\,L^{-3}$)
X_{10}: Cell concentration 10% higher than X_o ($M\,L^{-3}$)
μ: Maximum specific growth rate (T^{-1})
P: Volumetric productivity ($M\,L^{-3}\,T^{-1}$)
t_m: Time required to reach X_m (T)
t_o: Initial time (T)
t_{90}: Time required to reach X_{90} (T)
t_{10}: Time required to reach X_{10} (T)
Y: Microalgae yield coefficient; ratio of biomass amount produced per amount of substrate consumed ($M\,M^{-1}$)
Y_i: Microalgae yield coefficient of inoculum ($M\,M^{-1}$)
S: Total nutrient concentration at an instant t ($M\,L^{-3}$)
S_o: Initial substrate concentration (nutrients in the culture medium) ($M\,L^{-3}$)
S_{To}: Initial amount of substrate (nutrients in the culture medium and nutrients in the inoculum) ($M\,L^{-3}$)
S_a: Assimilable substrate concentration at an instant t ($M\,L^{-3}$)
S_{ao}: Initial assimilable substrate concentration ($M\,L^{-3}$)
S_{na}: Unassimilated substrate concentration ($M\,L^{-3}$)
k: Kinetic constant ($M^{-1}\,L^{3}\,T^{-1}$)
μ_N: Maximum specific nitrogen uptake rate (T^{-1})
μ_P: Maximum specific phosphorus uptake rate (T^{-1})

2. Materials and methods

2.1. Microorganism

C. vulgaris (SAG 211-12), obtained from the Culture Collection of Algae (SAG), Göttingen University (Germany), was used in this study. It was incubated in synthetic culture medium Combo [20] at 20 ± 1°C under 250 μmol m^{-2} s^{-1} light intensity with 14 : 10 h L : D cycle. The inoculum was maintained in exponential growth.

2.2. Experimental set-up

The experiments were conducted in batch photobioreactors by using borosilicate Pyrex bottles (12.5 cm diameter × 14.5 cm height) with 2 L of culture, sealed with caps with three lines: one for the introduction of air, one for the air outlet, and the last one for sampling. The air, injected into the cultivation from the bottom, was enriched in a proportion similar to that of combustion gasses of fossil fuel power plants for natural gas (5% CO_2) [21] to ensure dissolved carbon availability for cultures, filtered through 0.2 μm and bubbled at a 1.25 L min^{-1} flow rate. *Chlorella sp*. has demonstrated the ability to tolerate exposure to a gas containing up to 20% CO_2 applied continuously in batch reactors to the culture [22]. Microalgae were grown at 20 ± 1°C with a photoperiod of 14 : 10 h L : D and an irradiance of 250 μmol m^{-2} s^{-1}, provided by eight fluorescent lamps (4 PHILIPS Master TLD 58W/840 Cool White and 4 SYLVANIA Grolux F58W/GRO-T8 Daylight) placed horizontally and parallel to the front side of the photobioreactor. Reactors were inoculated with 200 mL suspension of precultured cells, obtaining an initial biomass concentration of around 70 mg L^{-1}.

2.3. Culture media

Five different culture media have been tested in this work: (1) the commercial synthetic medium Combo two-fold (named as *SM_NO3*); (2) the modified Combo two-fold medium, which composition is identical to Combo two-fold medium excepting the use of NH_4Cl ($M = 2.00 \times 10^{-3}$) as the nitrogen source instead of $4NaNO_3$ (*SM_NH4*); (3) urban wastewater from a secondary effluent of a conventional wastewater treatment plant (Arcos de la Frontera WWTP, 25,000 inhabitants) filtered through a glass fibre filter of 0.45 μm pore diameter (*WW*); (4) wastewater cited in (3) enriched in $NaNO_3$ and K_2HPO_4 to make the nitrogen and phosphorus final concentration similar to that of the Combo two-fold medium (*WW + NP*); and (5) modified wastewater described in (4) with the addition of micronutrients (minerals, metals and vitamins) from synthetic medium Combo two-fold (those which appear in the recipe of the Combo two-fold medium with an asterisk*) (*WW + NP + M*). In the WWTP the wastewater was submitted to pre-treatment, primary settling, activated sludge and final settling. Once nutrients were added (if necessary), media sterilisation was carried out using autoclaving for 20 min at 120°C and 1 kg cm^{-2}. Five identical culture media to those used in the experiment without any algal inoculation were used as controls. They were also kept under the same conditions as those with algae

Table 1. Characteristics of the five culture media.

Parameters	*SM_NO3*	*SM_NH4*	*WW*	*WW + NP*	*WW + NP + M*
NH_4-N (mg L^{-1})	<0.05	29.79	10.71	10.58	10.43
NO_3-N (mg L^{-1})	26.65	0.86	5.39	14.84	14.35
NO_2-N (mg L^{-1})	<0.02	<0.02	2.25	2.13	2.31
NH_4-N : (NO_3-N + NO_2-N)	0	35	1	1	1
PO_4-P (mg L^{-1})	2.99	2.77	0.52	2.53	2.53
N : P	9	11	35	11	11
COD (mgO_2 L^{-1})	<25	<25	70	70	70
pH	7.89	7.89	8.36	8.36	8.36
Conductivity (μS cm^{-1})	663	663	877	877	877

in flasks. The recipe of the Combo two-fold medium is (M): $NaNO_3$ (2.00×10^{-3}), $CaCl_2 \cdot 2H_2O^*$ (5.00×10^{-4}), $MgSO_4 \cdot 7H_2O^*$ (3.32×10^{-4}), $NaHCO_3^*$ (3.00×10^{-4}), K_2HPO_4 (1.00×10^{-4}), $H_3BO_3^*$ (7.76×10^{-4}), KCl^* (2.00×10^{-4}), $Na_2EDTA \cdot 2H_2O^*$ (2.34×10^{-5}), $FeCl_3 \cdot 6H_2O^*$ (7.40×10^{-6}), $CuSO_4 \cdot 5H_2O^*$ (8.02×10^{-9}), $ZnSO_4 \cdot 7H_2O^*$ (1.53×10^{-7}), $CoCl_2 \cdot 6H_2O^*$ (1.01×10^{-7}), $MnCl_2 \cdot 4H_2O^*$ (1.82×10^{-6}), $Na_2MoO_4 \cdot 2H_2O^*$ (1.82×10^{-7}), $H_2SeO_3^*$ (2.48×10^{-8}), $Na_3VO_4^*$ (1.96×10^{-8}), thiamine·HCl* (B_1) (5.92×10^{-7}), biotin* (vitamin H) (4.10×10^{-9}), cyanocobalamin (vitamin B_{12})* (8.12×10^{-10}). Chemical composition of the five media is listed in (Table 1).

2.4. Analytical methods

Temporal evolution of the microalgal biomass was measured daily, indirectly, by means of optical density at $\lambda = 680$ nm. A linear regression equation was developed between the optical density and the algal dry weight (suspended solids, mg SS $L^{-1} = (OD_{680} - 0.0334)/0.0029$; $R^2 = 0.99$). The algal dry weight was determined gravimetrically according to Standard Methods 2540-D [23].

Nutrient consumption was monitored on the samples. Daily samples withdrawn from flasks were filtered (0.45 μm nominal pore glass fibre filter) to separate the algae. As organic nitrogen and organic phosphate in the culture media were below detection limits (0.5 mg N L^{-1} and 0.044 mg P L^{-1}), total phosphorus was determined during the test as phosphate and total nitrogen as the sum of nitrate, nitrite and ammonium. Ammonia (4500-NH_3 D), nitrite (4500 NO_2^- B), phosphate (4500-P E) and total phosphorus (4500-PB4, 4500-P E) were determined colorimetrically according to standard methods [23]. Determination of nitrate was performed according to the colorimetric method 1.14773.0001 by Merck-Chemicals, where in concentrated sulfuric acid nitrate ions react with a benzoic acid derivative to form a red nitro compound that is determined photometrically. Total nitrogen was measured as nitrate after digestion using Oxisolv®, Merck-Chemicals.

The tests ended when the growth finished or the increase in biomass was lower than 1% per day. Then the cultures were harvested with a centrifuge and the biomass was dried in a lyophilizer (Labconco, FreeZone Triad Cascade Benchtop). The carbon, hydrogen, nitrogen and sulfur content of the dry biomass were measured in duplicate using an elementary analyser (LECO CHNS-932, Leco Corporation).

An acid digestion of the dry biomass was done in triplicate in a microwave digester (ETHOS 1600, Milestone) and total phosphorus was determined by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES, Iris intrepid, Thermo Elemental).

Lipids of the biomass were determined in duplicate. Lipids were extracted according to a modified method reported by [24] and [25]. To 90 mg of lyophilized pellets, 12 mL of 2 : 1 trichloromethane : methanol mixture and 0.6 g of analytical grade quartz were added and the mixture was sonicated in a bath (60 kHz; 360 W) for 90 min. Extraction was done twice and both extracts were mixed, centrifuged and filtered to ensure quartz separation. The filtrate was evaporated under reduced pressure in a rotary evaporator. The remainder was dried at $100 - 105$°C for 12 h and weighed as the total lipid.

2.5. Statistical analysis

The analysis of the data was performed using STATISTICA software (StatSoft, Inc. Version 7.0, 2004). The Quasi-Newton method was used for non-linear regression with a 10^{-4} convergence criterion.

3. Results and discussion

3.1. Culture, pH and controls

The initial pH of algal cultures was around 8.4 for the first two hours and suffered an initial decrease due to the air enriched in CO_2. This effect was more pronounced in reactors containing synthetic medium (pH $\approx$ 6) than in reactors containing wastewater (pH $\approx$ 7). During the experiment the pH was around 6.9 ± 0.1 in wastewater culturing media reactors and 6.3 ± 0.1 in the *SM_NO3* reactor. These differences in pH evolution are due to the higher buffering capacity of wastewater; distilled water used in the synthetic medium presented a carbonate ion content eight-fold lower than wastewater.

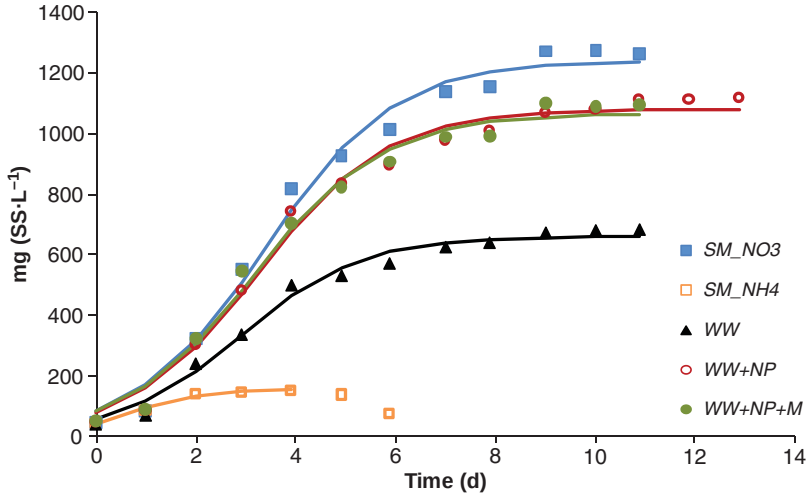

Figure 1. Biomass evolution of *C. vulgaris* in different culture media. Symbols are experimental data and solid lines depict the predicted data.

The microalgae cultured in *SM_NH4* suffered a decrease in the pH, reaching pH < 3 at 48 hours and did not rise again. The reason for this extreme pH is H^+ releasing from NH_4^+ during the metabolic utilisation of the NH_4^+ ion as nitrogen source as shown in Equation (1) [26]:

$$NH_4^+ \leftrightarrow NH_3 + H^+ \rightarrow \text{org-N} + H^+ \quad (1)$$

This reaction also occurred in wastewater, but in this medium the ammonium load was lower and besides, the wastewater presented a higher buffering capacity than the synthetic medium.

Except for in the reactor *SM_NH4*, which pH followed the same trend than the *SM_NO3* reactor, the pH in the controls followed the same evolution than inoculated reactors, which means that the CO_2 added controlled the pH instead of the microalgal activity. Photosynthetic activity leads to a gradual rise in pH, measuring pH 11 [27].

In the controls, algal contamination was not found and the nutrient content as well as the suspended solids did not change significantly during the experiments.

3.2. Biomass growth

Figure 1 shows the growth of *C. vulgaris* in the different culture media. The tests ended once the stationary phase was reached. Growth curves are different depending on the culture medium, except for *C. vulgaris* cultured in $WW + NP$ and in $WW + NP + M$, where the growth curves are similar. According to the results, *C. vulgaris* reached the higher biomass concentration in *SM_NO3* (1270 mg SS L^{-1}), slightly higher than in $WW + NP$ and $WW + NP + M$ (1116 mg SS L^{-1}). The growth in *WW* is lower (685 mg SS L^{-1}) and in *SM_NH4* hardly there is growth (151 mg SS L^{-1}). As depicted in Figure 1, this microalga can grow well in wastewater and to a higher extent when the wastewater is enriched in nitrogen and phosphorus.

SM_NH4 is not a suitable culture medium for *C. vulgaris* as presents little tolerance to pH values < 4 [28] and its growth is inhibited due to the low pH in the reactor (Figure 1). Therefore, to grow *C. vulgaris* in this culture media a pH buffer should be used to keep pH closer to 7, the optimal for the growth and lipid accumulation [29].

To quantify the growth, experimental data was described by the Verhulst model [18], since it was fitted to this logistic model being the variance explained in all the regressions > 98%. The results are plotted in Figure 1 as solid lines. Expressed mathematically:

$$\frac{\delta X(t)}{\delta t} = \mu X(t)\left[1 - \frac{X(t)}{X_m}\right] \quad (2)$$

The integrated form of the equation subject to initial value $X = X_o$ ($t = 0$) (Equation (2)) was used to describe the experimental data (Equation (3)):

$$X = \frac{X_o X_m e^{\mu t}}{X_m - X_o + X_o e^{\mu t}}. \quad (3)$$

In order to compare results between experiments, a useful parameter is volumetric productivity (P) defined as the biomass produced per reactor volume and per unit time. This parameter can be calculated from the kinetic parameters of Verhulst model as Equation (4):

$$P = \frac{X_m - X_o}{t_m - t_o}. \quad (4)$$

From Equation (3), an expression for time as function of biomass concentrations can be obtained (Equation (5)):

$$t = \frac{1}{\mu} \ln\left(\frac{X(X_m - X_o)}{X_o(X_m - X)}\right). \quad (5)$$

In this expression, t_m is equal to infinite, as X_m is the superior asymptotic value of the sinusoidal growth curve.

Table 2. Kinetic parameters of *C. vulgaris* growth from Verhulst model. The confidence intervals are derived from standard error from non-linear regression.

Parameters	*SM_NO3*	*SM_NH4*	*WW*	*WW + NP*	*WW + NP + M*
X_o (mg SS L^{-1})	83 ± 22	42 ± 7	56 ± 13	77 ± 17	84 ± 21
X_m (mg SS L^{-1})	1239 ± 31	155 ± 7	664 ± 15	1080 ± 19	1067 ± 26
μ (d^{-1})	0.78 ± 0.08	1.43 ± 0.28	0.83 ± 0.09	0.79 ± 0.07	0.78 ± 0.08
Productivity (g SS L^{-1} d^{-1})	0.17	0.04	0.10	0.15	0.15
R	0.99	0.99	0.99	0.99	0.99

So, in order to estimate productivity and instead of t_m and X_m, we will consider the very similar values t_{90} and X_{90}, i.e. the time required to reach 90% of the maximum biomass concentration.

When productivity in a batch reactor has to be calculated, the lag phase should not be considered because it is an extremely variable growth phase that depends not only on the environmental conditions of the experiment but also on the experimental methodology like initial inoculum concentration used and how this inoculum was obtained. In order to avoid this source of variation productivity should be calculated once the lag phase has finished. Many OECD guidelines to test biodegradability of chemicals [30] establish that the lag phase finishes once 10% of biodegradation has been reached. In this study, it could be comparable to a 10% increase of initial biomass concentration in the reactor (X_{10}).

According to this, productivity in the exponential growth phase in a batch photobioreactor could be approximated to (Equation (6)):

$$Productivity = \frac{X_{90} - X_{10}}{t_{90} - t_{10}} = \frac{0.9X_m - 1.1\,X_o}{t_{90} - t_{10}}. \quad (6)$$

Using Equation (5), the following is obtained:

$$t_{90} = \frac{1}{\mu} \ln\left(\frac{9(X_m - X_o)}{X_o}\right). \quad (7)$$

$$t_{10} = \frac{1}{\mu} \ln\left(\frac{1.1\,(X_m - X_o)}{X_m - 1.1\,X_o}\right). \quad (8)$$

Equation (9) is made by combining Equations (6), (7) and (8). This formula can be used to calculate the productivity of a batch photobioreactor from the kinetics parameter of Verhulst growth model in the exponential growth phase, defined as the time elapsed between an increase of biomass concentration of 10% of the initial and 90% of the maximum reached in the reactor at the end of the stationary phase.

$$Productivity = \frac{\mu(0.9X_m - 1.1\,X_o)}{\ln\left(\frac{9(X_m - 1.1\,X_o)}{1.1\,X_o}\right)}. \quad (9)$$

The kinetic parameters obtained from the model are listed in (Table 2).

According to [31], the average μ for freshwater species is 0.83 d^{-1}, similar to those obtained in this study (Table 2) with the exception of *SM_NH4*, where the maximum specific growth rate has been higher before *C. vulgaris* became inhibited, probably due to ammonium is the preferred nitrogen source [8,32,33].

Reactors *WW + NP* and *WW + NP + M* presented very similar maximum biomass concentrations and maximum specific growth rates (Table 2). Therefore, productivities are almost the same (0.15 g SS L^{-1} d^{-1}) showing that the addition of vitamins, minerals and metals to wastewater does not increase the growth of *C. vulgaris*. This indicates that urban wastewater effluents contain all the essentials oligoelements needed to culture *C. vulgaris*. The addition of these substances is not necessary to grow this microalga in wastewater until the concentration obtained in this study.

The microalga cultured in wastewater (*WW*) showed a lower productivity (0.10 g SS L^{-1} d^{-1}) than wastewaters enriched (0.15 g SS L^{-1} d^{-1}) (Table 2). This is due to nitrogen and phosphorus content in enriched wastewater was 1.5 and 5 times higher, respectively, than in wastewater.

Productivity in *SM_NO3* is the highest (0.17 g SS L^{-1} d^{-1}), slightly greater than in *WW + NP* and *WW + NP + M* (0.15 g SS L^{-1} d^{-1}). This difference in productivities is quite small (11.8%), however in Figure 1 the difference in the growth can be clearly observed. The reason could be the different chemical forms of nitrogen compounds in culture media. Nitrogen in *SM_NO3* is completely in the form of nitrate and in *WW + NP* and *WW + NP + M* it is as NO_3^- + NO_2^- (62%) and NH_4^+ (38%). As ammonium is the most preferred nitrogen source for algae, this presence of ammonium should not cause this lower value in the productivity. Wastewater is a complex mixture of chemicals that could cause toxicity to algae and affect to their maximum specific growth rate [34]. Therefore an inhibitory effect could be dismissed, as maximum specific growth rates are almost the same in these three reactors. The different productivity is a result of the maximum biomass concentration achieved. This larger biomass could be reached because of the slightly higher phosphorus concentration in the synthetic medium (Table 1), total phosphorus is a 15% higher in *SM_NO3* and the difference in productivities is about 12%.

According to this we can state that effluents from urban wastewater treatment plants are a suitable culture medium to grow microalgae, and comparable to synthetic medium. The subsequent use of the biomass could limit the use of wastewater, being irrelevant when biomass is cultured for energetic purposes.

The culture *SM_NH 4* presented a very low productivity (Table 2), caused by the inhibitory effect of acid pH in algal culture, which was 2.8 when the growth finished (Figure 1).

3.3. PhBT model for nutrient uptake by microalgae in batch photobioreactors

Considering a discontinuous reactor to which a certain amount of substrate (i.e. nitrogen and phosphorous) is added, an initial slow conversion of these nutrients into microalgae biomass will be observed. Because microalgae multiply during the course of the process, the reaction rate increases until a maximum is reached. Then, when most of the substrate is consumed, biomass growth gradually decreases and the substrate consumption rate falls to zero. Observing the following reaction, this is clearly an autocatalytic behaviour (Equation (10)):

$$Nutrients + X \rightarrow \alpha Products + \left(\frac{1}{Y}\right) X. \quad (10)$$

According to the stoichiometry of the reaction, for each molecule of nutrient consumed, α molecules of products are formed (O_2, excreta, extracellular enzymes, etc.), and $1/Y$ microalgae are produced.

The substrate consumption rate will be:

$$\frac{-\delta S_a}{\delta t} = k\, S_a\, X \quad (11)$$

If the initial concentration of nutrients in the reactor that will be incorporated by microalgae during the batch process is S_{ao} and the initial biomass concentration is X_o, after a time t when part of the substrate has been consumed, the concentration of assimilable nutrients remaining in the medium is S_a. Then, the concentration of microorganisms at that time (X) is (Equation (12)):

$$X = X_o + Y(S_{ao} - S_a). \quad (12)$$

Substituting this for X in the rate equation (Equation (11)) gives Equation (13):

$$\frac{-\delta S_a}{\delta t} = kS_a(X_o + Y(S_{ao} - S_a)). \quad (13)$$

Rearranging terms gives Equation (14):

$$\frac{-\delta S_a}{\delta t} = k\, Y\left[\left(\frac{X_o}{Y} + S_{ao}\right) S_a - S_a^2\right]. \quad (14)$$

But when batch photobioreactor experiments are conducted, the substrates analysed are not the assimilable nutrients (S_a) but the total nutrient concentration (S), being:

$$S = S_a + S_{na}. \quad (15)$$

$$S_o = S_{ao} + S_{nao} = S_{ao} + S_{na}. \quad (16)$$

Substituting Equations (15) and (16) into Equation (14), Equation (17) is obtained:

$$-\left(\frac{\delta S}{\delta t} - \frac{\delta S_{na}}{\delta t}\right) = k\, Y\left[\left(\frac{X_o}{Y} + (S_o - S_{na})\right)(S - S_{na}) - (S - S_{na})^2\right]. \quad (17)$$

Considering that the unassimilated substrate remains constant in the reactor and rearranging terms results, the resulting Equation (18) is:

$$\frac{-\delta S}{\delta t} = k\, Y\left[-S^2 + \left(\frac{X_o}{Y} + S_o + S_{na}\right) S - \left(\frac{X_o}{Y} + S_o\right) S_{na}\right]. \quad (18)$$

Considering that $(X_o/Y) + S_o = S_{To}$, i.e. the initial total amount of substrate including not only the nutrients in the culture medium (S_o) but also those in the initial inoculum (X_o/Y), then:

$$\frac{-\delta S}{\delta t} = k\, Y[-S^2 + (S_{To} + S_{na})S - S_{To}S_{na}]. \quad (19)$$

In Equation (19), the substrate consumption kinetics follows a quadratic equation. Separating the variables and integrating the equation, gives the relationship between substrate concentration in the batch photobioreactor and time as follows in Equations (20) and (21):

$$\int_0^t \delta t = \int_{So}^{S} \frac{\delta S}{k\, Y[-S^2 + (S_{To} + S_{na})S - S_{To}S_{na}]}. \quad (20)$$

and therefore:

$$S = \frac{h(S_o - q) - q(S_o - h)e^{pt}}{(S_o - q) - (S_o - h)e^{pt}}. \quad (21)$$

where:

$$q = \frac{k\, Y((S_{To} + S_{na}) - \sqrt{k^2Y^2(S_{To} + S_{na})^2 - 4k^2Y^2S_{To}S_{na}})}{2k\, Y}. \quad (22)$$

$$h = \frac{k\, Y((S_{To} + S_{na}) + \sqrt{k^2Y^2(S_{To} + S_{na})^2 - 4k^2Y^2S_{To}S_{na}})}{2k\, Y}. \quad (23)$$

$$p = \sqrt{k^2Y^2(S_{To} + S_{na})^2 - 4k^2\, Y^2S_{To}\, S_{na}}. \quad (24)$$

Equations (22) and (23) are the solutions for the quadratic equation:

$$k\, Y[-S^2 + (S_{To} + S_{na})S - S_{To}S_{na}] = 0$$

The two possible solutions for Equation (24), i.e. when substrate consumption kinetic is equal to zero, are at the initial and final stages of the process. This is when in Equation (21), S is equal to S_{To} (h) and S_{na} (q), respectively.

As stated above $(X_o/Y) + S_o = S_{To}$, so Equation (21) can be rewritten now as:

$$S = \frac{\left(\frac{X_o}{Y} + S_o\right)(S_o - S_{na}) - S_{na}\left(S_o - \left(\frac{X_o}{Y} + S_o\right)\right)e^{pt}}{(S_o - S_{na}) - \left(S_o - \left(\frac{X_o}{Y} + S_o\right)\right)e^{pt}}. \tag{25}$$

Considering Equations (26), (27) and (28):

$$(S_{To} - S_o) = \frac{X_o}{Y}. \tag{26}$$

$$(S_o - S_{na}) = \frac{(X_m - X_o)}{Y}. \tag{27}$$

$$\begin{aligned} S_a &= S_{ao} - \left(\frac{X - X_o}{Y}\right) \rightarrow S - S_{na} \\ &= (S_o - S_{na}) - \left(\frac{X}{Y}\right) + \left(\frac{X_o}{Y}\right) \rightarrow S \\ &= S_{To} - \left(\frac{X}{Y}\right) \end{aligned} \tag{28}$$

so then Equation (25) can be transformed into Equation (29):

$$S_{To} - \left(\frac{X}{Y}\right) = \frac{S_{To}\left(\frac{(X_m - X_o)}{Y}\right) + S_{na}\left(\frac{X_o}{Y}\right)e^{pt}}{\left(\frac{(X_m - X_o)}{Y}\right) + \left(\frac{X_o}{Y}\right)e^{pt}}. \tag{29}$$

Rearranging terms:

$$X = \frac{X_o X_m e^{pt}}{X_m - X_o + X_o e^{pt}}. \tag{30}$$

Equation (30) is the integrated form of Verhulst logistic kinetic biomass growth equation (Equation (3)); therefore p represents the maximum specific growth rate (μ) of the microalgae in the reactor.

Wide variations in the chemical composition of algae can occur at different stages of growth, with lower nutrient contents in older cells when these become scarce or are depleted from the medium [35]. Previous studies [36] have demonstrated variations in biomass composition throughout the study: decreasing nitrogen content at the end of the experiment, and varying the proportion of nitrogen from 1 to 10% [37]. Therefore, there is a change in nutrient distribution in the cells, and specifically, the inoculum in this experiment (obtained in exponential growth phase) presents a different composition than the biomass harvested at the end of the experiment (obtained in stationary phase) (Table 5 and Section 3.4.). When both nutrients studied are transported into the cell, they are stored in pools. With regard to phosphorus, three pools are present: internal pools of polyphosphate, soluble inorganic phosphorus and structural and other organic phosphorus [38]. With respect to nitrogen the pools are: internal pools of nitrate, ammonium and glutamine and other organic cellular nitrogen [39]. Microalgae sequester nutrients from the water column far beyond their immediate requirements and therefore inorganic pools increase. The microalgae yield coefficient (Y) is the ratio of biomass produced per mass of substrate incorporated as organic or structural. It can be determined analytically from biomass obtained at the end of the stationary phase, considering that after a continued nutrient depletion, almost all cellular nitrogen and phosphorus are present as structural pools rather than as inorganic pools [40–43]. So, Y can be calculated using Equation (31):

$$Y = \frac{(X_m - X_o)}{(S_o - S_{na})}. \tag{31}$$

3.4. Nutrient removal

As nutrient removal did not change significantly in the control experiments, nutrient removal is due to microalgal activity and not to physical or chemical effects as stripping of ammonium to ammonia gas or phosphorus precipitation result of the increase in pH [44].

Equation (25) has been used to describe the experimental data for total nitrogen and total phosphorus (Figures 2 and 3). Experimental data for nutrients were used as S value, X_o value applied was that from biomass growth modelling (Table 2) and the Y_i value of the inoculum was obtained experimentally. Inoculum composition was analysed, showing a nitrogen and phosphorus content of 7.18 and 0.74%, respectively. When applying the model, using the adjustable factors S_o, S_{na} and μ_N *or* μ_P, the variance explained was higher than 96% in all the experiments. The fitting was excellent and all the kinetic factors were logical values.

Therefore, this model seems to be adequate and presents kinetic parameters in common with the Verhulst model for biomass growth. This methodology could reduce the diversity of the data treatment used by different authors by setting a simple standard method for nutrient consumption by microalgae in batch operation.

The PhBT model is applicable to nitrogen and phosphorus, even if the studied nutrient is not limiting. Theoretically the model could be potentially applied to any nutrient available in a finite concentration, although it has not been experimentally demonstrated on this study.

As can be seen in Figure 2, the total nitrogen was almost completely removed due to fast uptake by algae in the first 70 culture h. It does not occur in the culture media *SM_NH*$_4$, where removal rate slows down with time and microalgae could not uptake more than 59% of the available nitrogen.

The removal of total phosphorus was very effective as can be seen in Figure 3. It has been completely removed in all the tests excepting in the culture media *SM_NH*$_4$, however in this test 64% of initial phosphorus was removed

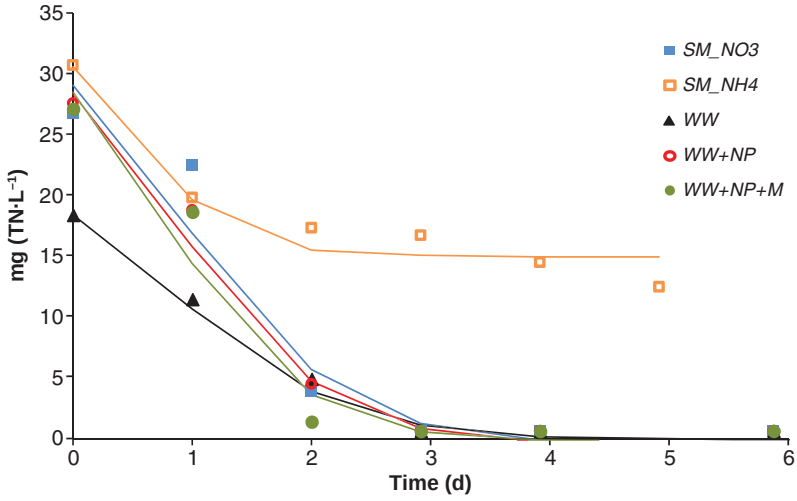

Figure 2. Total nitrogen uptake from different culture media by *C. vulgaris*. Symbols are experimental data and solid lines represent the nutrients predicted concentration according to the model.

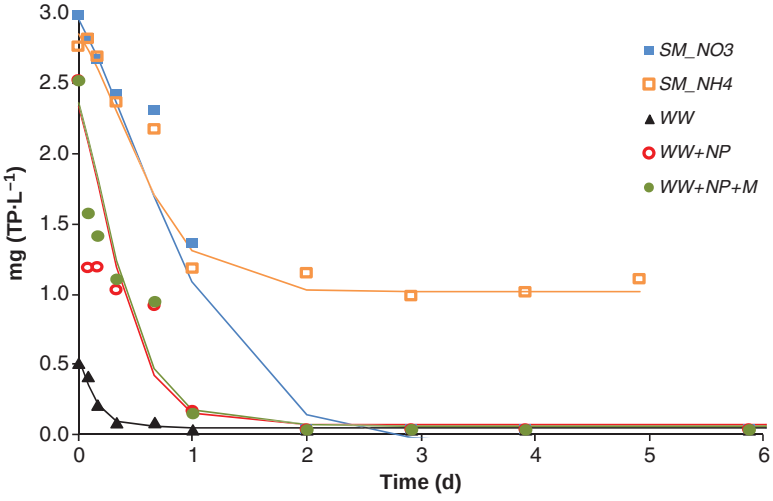

Figure 3. Removal of total phosphorus from different culture media by *C. vulgaris*. Symbols are experimental data and solid lines depict data predicted by the model.

despite the growth was inhibited. In *WW*, phosphorus is completely removed in 24 h.

The unassimilated substrate concentration (S_{na}) is quite low in all the experiments (Tables 3 and 4). It does not occur in the culture media *SM_NH*$_4$, where the pH inhibition stops the nutrient consumption and makes this value higher.

As demonstrated above, the maximum specific growth rates (μ) from the Verhulst model and the maximum specific growth rates (μ_N and μ_P) from the PhBT model (Tables 2, 3 and 4) are the same parameter, but when comparing them, it can be inferred that the values are different in the same experiments. The maximum specific growth rates obtained from the PhBT model are higher than the real ones. Those are the obtained from biomass growth modelling. The reason is the luxurious uptake of nutrients that are converted into inorganic storage products rather than structural and organic biomass. Therefore, the maximum specific growth rate from nutrient model can be used to estimate the nutrient consumption rate but not to predict biomass production because overestimation will occur. These parameters will be named as maximum specific nitrogen and phosphorus uptake rates (μ_N and μ_P) as they are related to nutrient consumption.

The specific phosphorus uptake rates are higher than those for nitrogen despite phosphorus content being lower in the biomass. It suggests that phosphorus uptake is higher than nitrogen in proportion to the biomass requirements. The data of [45] showed that luxury consumption of P was especially high, being stored in quantities up to 16 times the actual needs of *Selenastrum minutum*, whereas luxury uptake of N was lower than 4. Therefore the more luxury uptake is, the higher specific uptake rate is.

The highest maximum specific nitrogen uptake rate corresponds to the reactor *SM_NH*$_4$, 60% higher than in *SM_NO*$_3$. It is probably caused by the nitrogen source. In this culture medium ammonium is the only specie of

Table 3. Kinetic parameters of nitrogen removal obtained from PhBT model. The confidence intervals are derived from standard error from non-linear regression.

Parameters	*SM_NO3*	*SM_NH4*	*WW*	*WW + NP*	*WW + NP + M*
S_o (mg N L^{-1})	29.0 ± 3.1	30.5 ± 2.1	18.3 ± 0.6	28.3 ± 1.5	28.4 ± 2.5
S_{na} (mg N L^{-1})	−0.6 ± 2.4	14.9 ± 1.1	−0.2 ± 0.7	−0.7 ± 1.5	−0.4 ± 1.7
μ_N (d^{-1})	1.5 ± 0.3	2.5 ± 0.6	1.4 ± 0.1	1.6 ± 0.2	1.7 ± 0.3
Y (mg SS mg N^{-1})	39.0	7.3	32.8	34.5	34.1
R	0.98	0.97	0.99	0.99	0.99

Table 4. Kinetic parameters of phosphorus removal obtained from PhBT model. The confidence intervals are derived from standard error from non-linear regression.

Parameters	*SM_NO3*	*SM_NH4*	*WW*	*WW + NP*	*WW + NP + M*
S_o (mg P L^{-1})	3.0 ± 0.1	2.9 ± 0.1	0.5 ± 0.03	2.3 ± 0.2	2.4 ± 0.2
S_{na} (mg P L^{-1})	−0.1 ± 0.1	1.0 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
μ_P (d^{-1})	2.0 ± 0.2	3.2 ± 0.5	8.7 ± 1.2	4.4 ± 0.8	4.1 ± 0.9
Y (mg SS mg P^{-1})	383.0	61.9	1250.1	441.2	428.3
R	0.99	0.98	0.99	0.98	0.97

nitrogen available, which is the most energetically efficient source, since less energy is required for its uptake [8,46].

In cultures *SM_NO3*, *WW + NP* and *WW + NP + M*, there are no significant differences in specific nitrogen uptake rates. The values of this parameter are similar in the culture media *WW + NP* and *WW + NP + M* (1.6 and 1.7 d^{-1}, respectively), indicating that the extra metals, minerals and vitamins added do not significantly enhance nitrogen consumption.

Unlike what occurs with the nitrogen example, the maximum specific phosphorus uptake rates in wastewater are higher than those obtained in both synthetic media (*SM_NO3* and *SM_NH4*). This is probably due to the presence of different phosphorus species in wastewater besides phosphate, which could be adsorbed to the surface of the cells, which has been discussed previously by various authors [47].

The specific phosphorus uptake rates in the reactors *WW + NP* and *WW + NP + M* (4.4 and 4.1 d^{-1}, respectively) (Tables 3 and 4) indicate that phosphorus uptake is not enhanced by additional micronutrients.

When in the nutrient model (Equation (25)) the parameters S_o, S_{to} and S_{na} are replaced by the values obtained from nutrient depletion modelling and μ from biomass growth modelling is used, a curve of nutrient consumption over time is obtained. This is the theoretical nutrient used strictly in growth. Therefore, the difference between theoretical growth nutrients and measured nutrients can be used to estimate the luxurious uptake. In Figure 4a, this assertion is depicted for nitrogen as an example in the experiment *WW + NP + M*. The difference between these two curves is the estimated nutrient uptake and is not used instantly in producing new biomass. When this difference is divided by the momentary concentration of microorganisms (X), the amount of nutrient in excess per biomass can be estimated (Figure 4b, example for nitrogen in experiment *WW + NP + M*).

The maximum nitrogen and phosphorus concentrations in biomass in the tests are achieved in ranges between 0.4 and 1.8 d (data not shown), when nutrient consumption has started and biomass growth is not yet at a maximum. These maximum values in the experiments extend in a range between 9.5 and 50.0 μgN mgSS^{-1} and 1.5 and 12.0 μgP mgSS^{-1} (data not shown), meaning nitrogen and phosphorus reserves of up to a 5 and a 1.2% of the biomass.

As shown in Figure 4b, from a batch experiment the PhBT model can be used to estimate microalgae luxurious uptake of nutrient and the maximum content of nutrient reserves along the experiment. It is an important operation factor to consider performing the proper cultivation mode on wastewater treatment, where the main objective is the nutrient removal rather than maximising biomass production. It would be interesting in this kind of processes obtaining a maximum nutrient removal by harvesting a biomass rich in nutrients.

3.5. Microalgae yield coefficient

Microalgae yield coefficients (Y) for nitrogen and phosphorus have been calculated according to Equation (31) (Tables 3 and 4).

For nitrogen this coefficient is around 35 in all the experiments except for *SM_NH4*, where the stationary phase was not reached (Table 3). However, Y values for phosphorus show higher differences between experiments (Table 4). It suggests that nitrogen could be the limiting nutrient instead of phosphorus since at the stationary phase Y values for nitrogen are constant. The Redfield mass ratio of 41C : 7N : 1P [48] can be used as a general formula to estimate the nutrient limitation on the culture medium. Wastewater

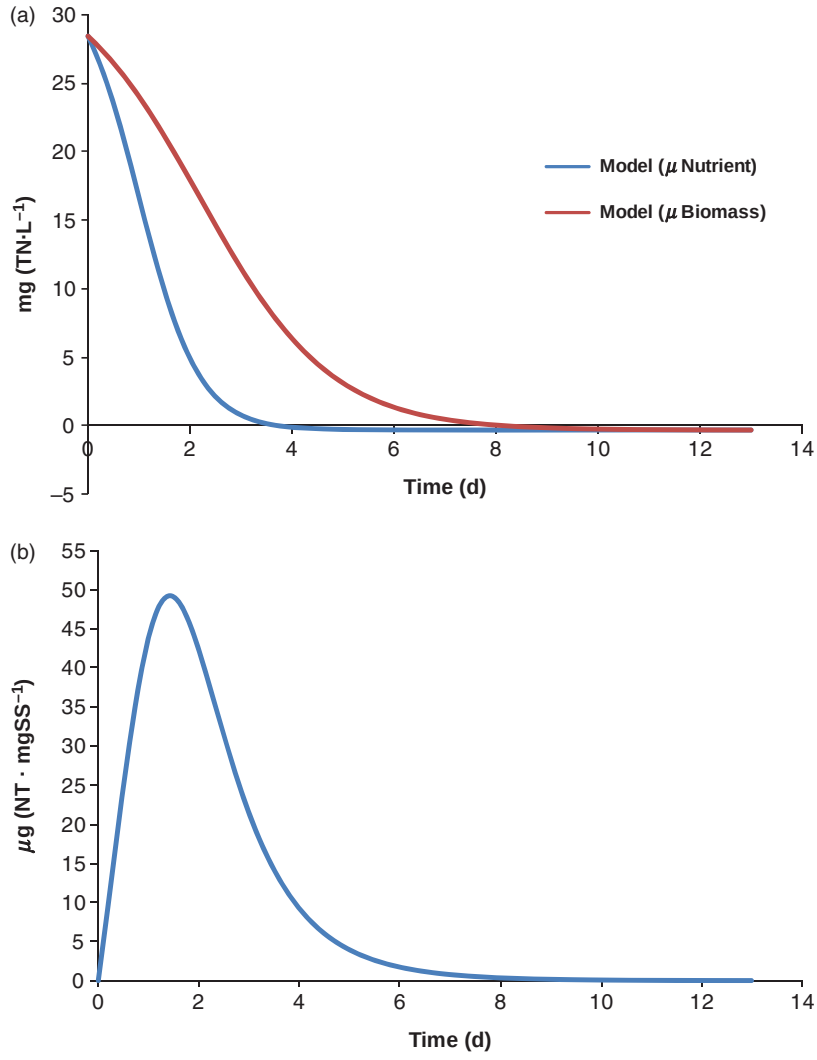

Figure 4. Experiment $WW + NP + M$. (a) Nitrogen predicted concentration according to PhBT model; comparison of using the specific growth rate from biomass growth and from nutrient model. (b) Estimated excess of nitrogen concentration in biomass.

contains an N : P ratio of 35 (Table 1), suggesting it is a phosphorus limited culture media, supporting the results of the microalgae yield coefficients. Enriched wastewaters and Combo media contains N : P ratios of 11 and 9, respectively (Table 1), close to the Redfield ratio, so we cannot state nitrogen limitation as this is an approximate value and can vary among species.

The nitrogen and phosphorus content in the biomass can be calculated as the inverse of the Y value. This estimated nutrient content was recorded as a percentage and the value is similar to those obtained experimentally when compared (Table 5).

3.6. *Compositional analysis*

Biomass obtained was analysed for its composition at the end of the tests, when the culture media were practically free of nitrogen and phosphorus (Figures 2 and 3). The low biomass concentration reached in SM_NH_4 impeded compositional analysis (Table 5).

The results of the elementary composition are similar between different cultures. Carbon values are analogous to that reported by [7] for microalgae cultured in wastewater; however nitrogen and phosphorus content are lower than that obtained by that author.

Though the N : P ratio of microalgae is not a useful indicator of the limiting nutrient, according to [35], N : P ratios lower than 10–20 can be an indication of growth limited by nitrogen. In the present study, biomass N : P ratios are very similar in $WW + NP$, $WW + NP + M$ and SM_NO_3 and lower than 9, which could suggest nitrogen is the limiting nutrient. However, microalgae cultured in WW presented less than half the phosphorus content than the rest of microalgae, suggesting limited phosphorus content in the WW, having a biomass N : P ratio of 17.5.

Table 5. Compositional analysis and lipid content of *C. vulgaris* after the experiments.

	SM_NO3	*WW*	*WW + NP*	*WW + NP + M*
C (%)	59.3 ± 0.03	52.3 ± 3.4	58.6 ± 0.2	53.8 ± 0.4
N (%)	2.7 ± 0.003	2.1 ± 0.1	2.3 ± 0.01	2.1 ± 0.02
N (%) predicted from the model	2.6	3.0	2.9	2.9
P (%)	0.31 ± 0.001	0.12 ± 0.001	0.29 ± 0.003	0.29 ± 0.001
P (%) predicted from the model	0.26	0.08	0.23	0.23
Lipids (%)	37.0 ± 0.9	45.7 ± 0.4	39.5 ± 1.0	37.2 ± 0.7
Lipid productivity (mg L^{-1} d^{-1})	62.4	45.2	59.1	55.3

3.7. Lipid content

The lipid content of the microalgae was determined when the tests had finished (Table 5). The lipid content varied between 37.0 (*SM_NO3*) and 45.7% (*WW*). These percentages agree with the average lipid content (42 %) for *C. vulgaris* cultured in a nitrogen deficient medium [31]. The reason for this high lipid content is that lipid accumulation occurs when a nutrient (usually nitrogen) is depleted from the medium [49] and in this work cell growth was observed after the disappearance of nitrogen and phosphorus from the medium. It is especially noticeable in *C. vulgaris* cultured in the most nutrient-poor medium, which showed the higher lipid content (45.7% in *WW*) (Table 5).

Lipid productivity is obtained as the product of percentage of lipids present in the biomass (Table 5) and biomass productivity (Table 2). This parameter is important in order to increase the economic feasibility of the microalgal culture for biodiesel production. Lipid productivities in *SM_NO3*, *WW + NP* and *WW + NP + M* are similar. Nevertheless, lipid productivity in *WW* is the lowest despite the lipid content is the highest since biomass productivity is much lower. Lipid productivities obtained agree with those collected by [31] from literature for *C. vulgaris*. Some authors [50] obtained comparable lipid productivities for *C. vulgaris* in artificial wastewater (44.79 and 147 mg L^{-1} d^{-1}).

The best medium to obtain maximum lipid production seems to be the synthetic medium Combo two-fold (Table 5), however enriched wastewater presented a lipid productivity slightly lower, achieving energetic and economic potential savings when wastewater is used.

4. Conclusion

This paper establishes a methodology that is able to reduce the diversity of the data treatment used by different authors, by setting a simple standard method. The PhBT model has been developed and successfully applied to describe the nutrient consumption by microalgae in batch operation. It is applicable to nitrogen and phosphorus, even if the studied nutrient is not limiting, giving some clues about which one is the limiting element and estimation about luxurious uptake.

Despite the low phosphorus content, the wastewater effluent could support a productive algal growth and efficient nutrient removal, supporting the idea of applying algal cultivation as a tertiary process in wastewater treatment plants.

Enriched wastewater has proven to be a suitable culture medium, presenting biomass productivity comparable to synthetic medium Combo, and even faster phosphorus removal.

Wastewater contains the vitamins and oligoelements needed to obtain the amount of biomass achieved, not improving the growth or nutrient consumption a further addition of these nutrients present in a synthetic medium.

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